Population genetics of the Amazonian tortoises, *Chelonoidis denticulata* and *C. carbonaria*, (Cryptodira: Testudinidae) in an area of sympatry

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Abstract. We conducted a population genetic analysis of the two Amazonian tortoises, *Chelonoidis denticulata* (n = 40) and *Chelonoidis carbonaria* (n = 39) in a region of sympatry within the Xingú River basin. High levels of gene flow among sampled localities indicated lack of population structure for both species. Genetic parameters indicated a moderate level of genetic diversity in *C. denticulata* and neutrality tests suggested that populations of this species were in demographic equilibrium with respect to mitochondrial DNA. On the other hand, *C. carbonaria* presented low levels of genetic diversity and a signal of population expansion. Most records of *C. denticulata* are from areas of humid forest while those for *C. carbonaria* are from areas of semi-deciduous forests and transitional areas between humid and semi-deciduous forests. Therefore, the demographic expansion observed in *C. carbonaria* population could reflect an increase in the availability of suitable habitats for this species due to anthropogenic or natural processes. Additionally, we observed haplotype sharing between these two tortoise species indicating hybridization or incomplete lineage sorting.

Keywords: Amazonian tortoises, *Chelonoidis* population genetics, cytochrome *b*, conservation.

Introduction

Chelonians inhabit several types of aquatic and terrestrial ecosystems, from oceans to rivers and lakes, and from deserts to tropical forests (Ernst and Barbour, 1989; Zug, 1993). The family Testudinidae is represented exclusively by terrestrial species, including more than 200 fossil forms (Auffenberg, 1974) and approximately 40 living species (Pough et al., 2001), and occurs in all continents except Australia and Antarctica (Zug, 1993). The genus *Chelonoidis* (Fitzinger, 1835) is represented by four species from South American and the Galápagos, if all tortoise forms from the Galápagos archipelago are considered sub-species of *C. nigra* (Iverson, 1992). *Chelonoidis* was previously considered a subgenus of *Geochelone* (Fitzinger, 1856),

but a recent molecular phylogenetic analysis of testudinids (Le et al., 2006), which represents the most comprehensive taxon sampling to date for this group, supports the elevation of *Chelonoidis* to generic status. Therefore we are following the newly proposed classification.

Three species of *Chelonoidis* occur in continental South America. *Chelonoidis chilensis*, the smallest species, has a more restricted geographical distribution, ranging from southwestern Bolivia, western Paraguay and northwestern Argentina to about 40°S in northern Patagônia, whereas *C. denticulata* and *C. carbonaria* are widespread throughout central and northern South America (Iverson, 1991; Jerozolimski, 2005). *Chelonoidis denticulata* and *C. carbonaria* are very similar in many aspects, including body size and shape, diet and behavior. Nevertheless, they can be reliably distinguished in the field using a set of coloration and morphological features (Williams, 1960; Castaño-Mora and Lugo-Rugeles, 1981; Pritchard and Trebbau, 1984; Moskovits, 1985; Moreira, 1991). *Chelonoidis denticulata* occurs mainly in moist tropical forests, whereas *C. car-*

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bonaria occurs in a wider range of habitats, including more open, drier forests and patches of forest vegetation in savannahs (Zug, 1993; Jerozolimski, 2005). However, in a number of localities these two species are sympatric, especially in transitional areas between forest and more open habitats such as savannahs (Medem et al., 1979; Pritchard and Trebbau, 1984; Moskovits, 1998; Jerozolimski, 2005).

The five contiguous reserves of the Kayapó Amerindians located in southern Pará and northern Mato Grosso encompass an area of approximately 113,000 km^2 of well preserved forests and cerrados (the Brazilian savannahs; see Eiten, 1972). They represent a barrier to the expansion of the colonization frontier from the east and to expected deforestation from the west following government plans to complete the pavement of the Cuiabá-Santarém highway. Whilst outside the Kayapó reserves most of the forests have been clear-cut and natural ecosystems have been severely altered, most of the Kayapó land is still covered by well preserved forests and cerrados and harbors many species considered sensitive to hunting pressure. Both *Chelonoidis denticulata* and *C. carbonaria* occur in this area and they are very important as a food resource for the Kayapó Amerindians. Between November 1994 and July 1996 the community of A'Ukre consumed a total of 350 tortoises, which represented 13% of all animal biomass consumed (Nascimento, 1997). Although they are intensively consumed, the combination of a low Kayapó population size spread over a large area (<0.07 inhabitant/km^2) probably explains the persistence of tortoise and other game populations in the area.

We evaluated the amount and distribution of genetic variability within naturally sympatric populations of *C. denticulata* and *C. carbonaria* using mitochondrial DNA (mtDNA) sequences from the cytochrome *b* gene. We inferred the hierarchical relationships between haplotypes and tested the relationship between genealogical lineages and the geographical distribution of sampled sites, and estimated when these two lin-

eages had diverged. The direct assessment of the amount and distribution of genetic variability within populations, combined with ecological field information, can increase our understanding of how wild populations of closely related species with different ecological requirements respond to changes in habitat availability and might provide important guidelines for management decisions.

Material and methods

This study was conducted in the Kayapó Indian Land, more specifically in the territories of the villages of A'Ukre and Moikarakô, located in the upper headwater regions of the Xingú River basin (Brazil), southern Pará state (fig. 1). Blood samples were collected in four different areas (fig. 1c) from tortoises obtained by Kayapó Indians during hunting trips. The site in the extreme north of the study area was located in the territory of the Moikarakô village (locality S4 in fig. 1c) whereas the other three sampled sites were located in the territory of the A'Ukre village.

Tortoises were identified to species (*C. denticulata* or *C. carbonaria*) using a group of characters described by Williams (1960), Castaño-Mora and Lugo-Rugeles (1981), Pritchard and Trebbau (1984) and Moskovits (1998), and marked individually with numbers painted on their carapaces with nail polish. Blood samples were obtained from harvested animals at the time they were killed (for consumption in the villages of A'Ukre and Moikarakô), and preserved in 95% ethanol. DNA extraction was done by dissolving and digesting the samples with a Proteinase K/SDS solution, followed by an addition of phenol-chloroform, a subsequent addition of 5M NaCl, and a final addition of 70% ethanol for DNA precipitation (Sambrook et al., 1989).

The initial 433 base pair of the mitochondrial cytochrome *b* (cyt*b*) gene were amplified by the Polymerase Chain Reaction (PCR) using the primers L14725 (Pääbo et al., 1990) and H15149 (Kocher et al., 1989), under the following protocol: denaturation at 94°C for 35 seconds, annealing at 50°C for 35 seconds, and extension at 72°C for 90 seconds repeated for 35 cycles. Sequencing reactions were performed according to the manufacturer's recommendation using the Terminator Cycle Sequencing Kit (Amersham Bioscience), and resolved on a MegaBACE automated sequencer (Amersham Bioscience).

Data analysis

Homologous protein-coding regions were aligned manually and confirmed by translating the DNA data into putative amino acid sequences using the program BioEdit (Hall, 1999). A number of statistical methods have been developed to infer historical processes shaping observed patterns of genetic distribution and diversity. The genetic equilibrium of mtDNA alleles was tested using Tajima's *D* test (Tajima,
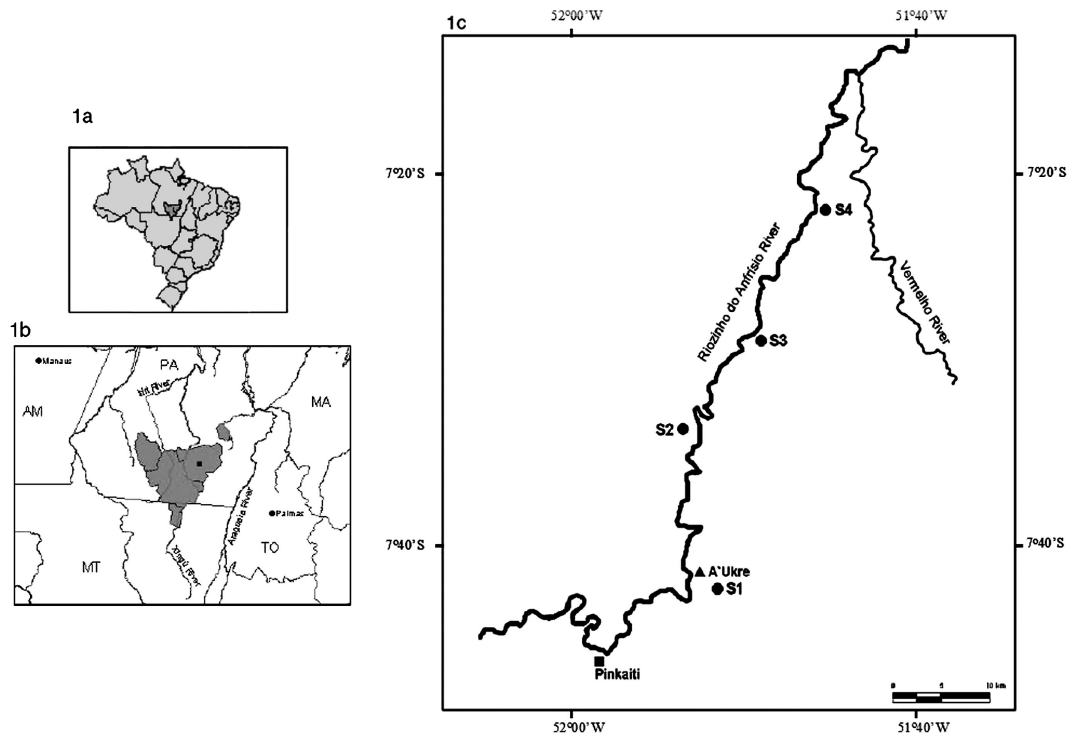
Figure 1. Location of study site. Kayapó Indian Lands are represented in dark grey in the maps on the left. The black square in the botton left map represents the study area, which is magnified in the right picture, showing the Kayapó village of A'Ukre (black triangle), the Pinkaiti research station (black square), and the location from where tortoises blood samples were obtained (S).

1989), and Fu's *Fs* test (Fu, 1997), which are based on the infinite-site model under the hypothesis of selective neutrality and population equilibrium. Although these tests have been formally designed to evaluate whether mutations are neutral or under influence of selection they also have been used to test for deviation from expectations of a population expansion model. We also calculate mismatch distribution as implemented in the program DnaSP (Rozas et al., 2003) where the distributions of pair-wise differences are compared with the expected distribution under a model of population expansion (Rogers and Harpending, 1992).

The presence of population subdivisions was tested using an analysis of molecular variance (AMOVA Excoffier et al., 1992), and a pair-wise population Φ_{ST} (analogues of F_{ST}) significance test as implemented in the program AR-LEQUIN (Excoffier et al., 2005). The level of genetic diversity was measured by the nucleotide diversity (per site) and gene (haplotype) diversity values which can be defined as the probability that two randomly chosen haplotypes are different in the sample (Nei, 1987). The gene diversity is equivalent to the expected heterozygosity for diploid data. The nucleotide diversity (π), which is the average number of base differences per site between two homologous sequences randomly selected from a population, is an important parameter used to understand the structure and history of populations. Nucleotide variability was also measured using the population parameter Theta (θ), which considers the

number of segregating sites (s) and the length of the sequences (i.e. the number of sites under study) and is one of the most important parameters in population genetics. The haplotype network for each species was reconstructed using the program TCS, version 1.13 (Clement et al., 2000), which implements the algorithm described by Templeton et al. (1992).

Results

Population genetics analysis

A total of 40 individuals of *C. denticulata* from four locations, and 39 individuals of *C. carbonaria* from three locations (all except S1) were analyzed. The 433 base pairs of the 5' end of the cytochrome *b* (cyt-*b*) sequences were used for population level studies of the two *Chelonoidis* species; this region contained a total of 47 variable positions (table 1). A total of eight haplotypes was found in *C. carbonaria* samples and five in *C. denticulata* samples. The fre-

Table 1. Forty seven variable sites in the 433 base pairs of the mitochondrial DNA cytochrome b of *Chelonoidis carbonaria* (*Cc*) and *Chelonoidis denticulata* (*Cd*) haplotypes. Identity with the first sequence is denoted by a dot. The numbers of individuals (N) for each haplotype are shown.

Haplotypes	Nucleotide Position	N
	0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 4	
	0 1 3 4 5 6 6 7 0 1 2 3 3 3 3 4 5 6 7 8 9 0 0 1 2 3 3 4 6 9 0 1 1 2 2 2 3 3 5 6 6 7 8 9 9 9 3	
	4 3 9 2 1 6 9 2 5 4 9 2 5 7 9 4 6 8 2 6 9 1 2 9 2 1 7 0 1 4 9 5 8 1 5 7 0 9 8 0 1 8 7 1 6 9 1	
*Cc*H01	A C G T C G C T A A T A A C G C A T T G A C G T G A C C A T T T T C C A T C A A T C C G T C C	30
*Cc*H02	. . . . T	02
*Cc*H03	. . . . C	01
*Cc*H04	. . . . A . . .	01
*Cc*H05	. . . . G	02
*Cc*H06	. . . . G . . . . G	01
*Cc*H07	. . . . G	01
*Cc*H08	. . . . T .	01
*Cd*H01[a]	G T A C . A A C G . A G . T A T G C C A G A A C A G T T G C . C C T T . C T T G C T T . C T ?	17
*Cd*H02	G T A . . A A C G . A G . T A T G C C A G A A C A G T T G C . C C T T . C T T G C T T . C T ?	01
*Cd*H03[b]	G T A C . A A C . . A G . T A T G C C A G A A C A G T T G C . C C T T . C . T G C T T . C T ?	18
*Cd*H04[c]	G T A C . A A C . . A G . T A T G C C A G A A C A G T T G C . C C T T G C . T G C T T . C T ?	01
*Cd*H05	G T A C . A A C . G A G . T A T G C C A G A A C A G T T G C . C C T T . C . T G C T T . C T ?	03

a – two individuals possessing haplotype *Cd*H01 were morphological *C. carbonaria*.
b – one individual possessing haplotype *Cd*H03 were morphological *C. carbonaria*.
c – one individual possessing haplotype *Cd*H04 were morphological *C. carbonaria*.

quency of each haplotype is shown at table 2. Sufficiently high levels of informative genetic variation provided us with a powerful tool for detecting genetic differentiation in these two South American tortoises.

The mean base frequency for *C. carbonaria* was 31% A, 27% T, 28% C and 14% G, and for *C. denticulata* was 32% A, 27% T, 27% C and 14% G, confirming an under-representation of guanine as is normally observed in all mitochondrial genome (Zhang and Hewitt, 1996). The nucleotide sequence data determined for the present work are deposited in GenBank (accession numbers: EF 490386-EF 490401).

High values of gene diversity and haplotype diversity (Nei, 1987) were observed in the samples of *C. denticulata* but not in *C. carbonaria* samples. The parameter θ was approximately 1 in all localities with the exception of sites 3 and 4 for *C. denticulata* and *C. carbonaria*, respectively. The main genetic patterns found in these analyses are summarized in table 3. These results suggest that these species may not have similar amounts of genetic diversity. However, the findings reflect only the current situation in

Table 2. Frequency of the haplotypes of *C. carbonaria* and *C. denticulata* in the four sampled areas.

	Haplotype	Site 1	Site 2	Site 3	Site 4	Total
Chelonoidis	*Cc*H01		5	17	8	30
carbonaria	*Cc*H02		2			2
	*Cc*H03			1		1
	*Cc*H04			1		1
	*Cc*H05		1		1	2
	*Cc*H06			1		1
	*Cc*H07			1		1
	*Cc*H08		1			1
	Total		9	21	9	39
Chelonoidis	*Cd*H01	2	1	9	5[a]	17
denticulata	*Cd*H02	1				1
	*Cd*H03	4	6	4	4[b]	18
	*Cd*H04				1[c]	1
	*Cd*H05	1	2			3
	Total	8	9	13	10	40

a – two individuals possessing haplotype *Cd*H01 were morphological *C. carbonaria*.
b – one individual possessing haplotype *Cd*H03 were morphological *C. carbonaria*.
c – one individual possessing haplotype *Cd*H04 were morphological *C. carbonaria*.

the sampled region. It is possible that different levels of genetic diversity may exist throughout the distributional range of these two species.

Table 3. Main genetics patterns for *C. carbonaria* and *C. denticulata*.

Species	Sampling	N	No. of haplotypes	Gene (Haplotype) Diversity	Nucleotide Diversity (per site) – π	Θ from s	Tajima's D test	Fu's Fs test
C. denticulata	Site1	8	4	0.750 ± 0.14	0.004	0.0036	0.081	−0.329
	Site2	9	3	0.556 ± 0.16	0.002	0.0026	−0.936	0.016
	Site3	13	2	0.462 ± 0.07	0.002	0.0015	1.214	2.300
	Site4	10	3	0.644 ± 0.10	0.003	0.0025	0.850	1.021
	All	40	5	0.626 ± 0.04	0.003	0.0027	0.177	0.221
C. carbonaria	Site2	9	4	0.694 ± 0.15	0.002	0.0026	−0.936	−1.417*
	Site3	21	5	0.352 ± 0.13	0.001	0.0026	−1.653*	−3.127*
	Site4	9	2	0.222 ± 0.16	0.0004	0.0008	−1.088	−0.263
	All	39	8	0.410 ± 0.10	0.001	0.0033	−1.739	−6.795*

Note: N = number of individuals; significance level: 5%.

In *C. carbonaria*, a total of eight haplotypes separated by six segregating sites were found; one haplotype predominates while the others where rare haplotypes or singletons (table 1; fig. 2a). On the other hand, *C. denticulata* had five haplotypes separated by five segregating sites, with two common haplotypes shared by all populations (table 1; fig. 2b). The uncorrected within species sequence divergence (uncorrected "*p*" distance) ranged from 0.0% to 0.6% (average 0.2%) for *C. denticulata*, and from 0.0% to 0.4% (average 0.2%) for *C. carbonaria*. The average sequence divergence between these two species was 9.0%. Analysis of Molecular Variance (AMOVA) suggested that there was no significant population structuring ($P > 0.05$). The mean source of genetic variation was attributable to variance within regions (94% for both species), and the gene flow among regions was high (tables 4 and 5).

Only in *C. carbonaria* Tajima's D statistic test and Fu's Fs test were significantly negative (table 3), i.e. both tests showed an excess of the number of segregating sites compared to the average pair-wise sequence divergence. These results were observed in two out of three sampled areas, and also when we considered all the sampled sites together. Both tests are negative under an excess of recent mutations and a significantly negative value is taken as evidence of population growth and/or selection (Tajima, 1989; Fu, 1997). This pattern is frequently observed in populations undergoing a rapid demographic expansion. For *C. denticulata* Tajima's test and Fu's Fs were not significant, which implies that the populations were at a mutation-drift genetic equilibrium with regard to the mitochondrial DNA haplotypes. These two species also showed different mismatch distributions (fig. 3); *C. denticulata* data showed a multimodal distribution characteristic of demographically stable populations, while *C. carbonaria* data presented a curve that fits the expected distribution of an expanding population.

Discussion

Chelonoidis denticulata and *C. carbonaria* are considered threatened under CITES Appendix II, which means that the "species are not necessarily threatened with extinction but that may become so unless trade is closely controlled". In addition, *G. denticulata* is considered Vulnerable in the IUCN (The World Conservation Union) Red List, which translates into "facing a high risk of extinction in the wild" (IUCN, 2004). Assignments into these categories are based on few data. Lack of information on tortoise populations in their natural habitats together with the fact that the study area is located in one of the least studied regions in the Amazon highlights the importance of this study.

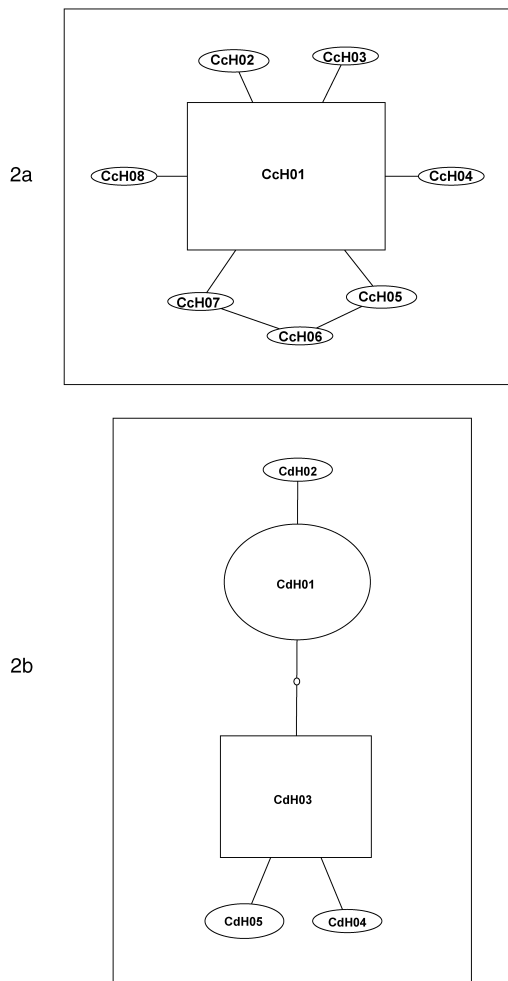Our results indicated high levels of gene flow and connectivity among the four sampled lo-

Figure 2. A haplotype network for Cytochrome *b* of *C. car-bonaria* (2.A) and *C. denticulata* (2.B). Each oval represents a haplotype and the size of the oval is proportional to the number of individuals of that particular haplotype.

Table 4. Matrix of pair-wise Φ_{ST} values (lower) and *Nm* values (upper) between *C. denticulata* populations.

Population	Site1	Site2	Site3	Site4
Site1	–	23.268	5.038	infinity
Site2	0.0210	–	0.691	2.197
Site3	0.0903	0.4197	–	infinity
Site4	−0.0657	0.1854	−0.012	–

Note: Significance level: 0.8% (after Bonferroni correction).

Table 5. Matrix of pair-wise Φ_{ST} values (lower) and *Nm* values (upper) between *C. carbonaria* populations.

Population	Site2,3	Site4	Site5
Site2	–	8.4	85.8
Site3	0.0561	–	infinity
Site4	0.0058	−0.0362	–

Note: Significance level: 5%.

more than 3.5 and 4.0 km away from the location they were first observed. Results of the site fidelity test performed in the Arc View program (version 3.2; Environmental Systems Research Institute, Inc.) using the "Animal Movement" extension (Hooge and Eichenlaub, 1997) indicated that from a total of 26 individuals of *C. denticulata* which were monitored for more then 20 days, seven were dispersing (Jerozolimski, 2005). Strong and Fragoso (2006) found similarly high rates of movement in both species studied in Maracá, a rainforest – cerrado ecotone in the Brazilian state of Roraima.

Furthermore, although tortoises do not present any evident morphological adaptation for swimming, individuals of both species were observed swimming across rivers in the central Amazon. For instance, M.N.F. da Silva (cited in Censky, 1988) mentioned the occurrence of tortoises floating in the Amazon River, and Castaño-Mora and Lugo-Rugeles (1981) observed the same in the Meta River in Colombia. The same behavior was recorded by M. Martins in the Uatumã River (AM, Brazil) and by A. Jerozolimski in the Riozinho do Anfrísio River in our study area. All of these records suggest that river systems could further increase gene flux between regions. The observed high levels of gene flow suggest that both species probably have a high
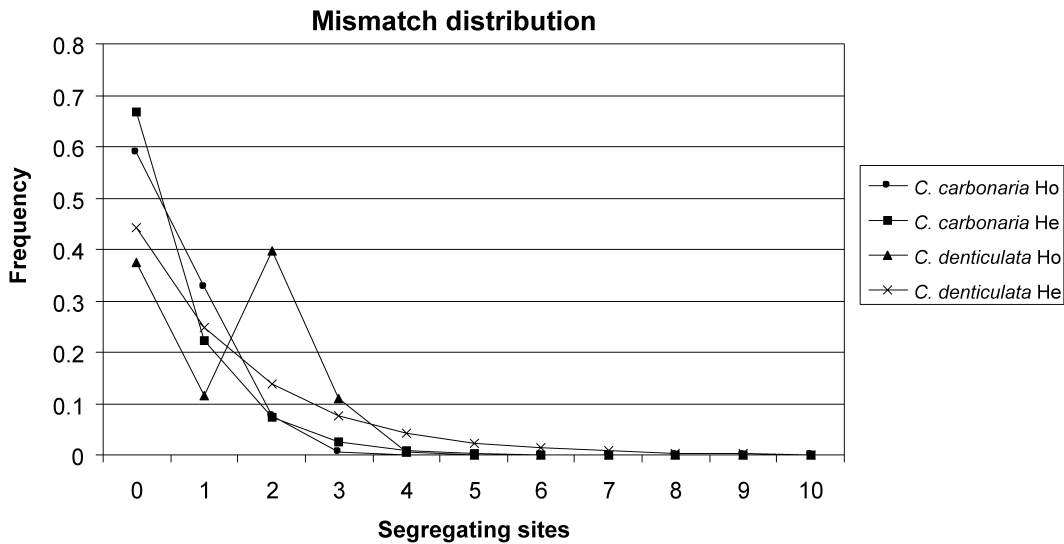
calities. No differentiation among localities or structuring of genetic information across geographic space was apparent in either tortoise populations.

Data on movement capacity obtained from radio tracked tortoises at the Pinkaiti reserve, an area in close geographic proximity to our sampling sites (fig. 1), supported the results of the population genetic analysis, indicating that dispersal capacity between regions is high and, therefore, there is a high gene flow potential (see tables 4 and 5). Individuals of *C. carbonaria* and *C. denticulata* were recorded, respectively,

Mismatch distribution

Figure 3. Mismatch distribution for all samples of *C. carbonaria* (a) and *C. denticulata* (b).

capacity to colonize newly available habitats and to recolonize areas where populations were depleted by hunting.

Four individuals from Site number 04 (Moikarakô) identified as *C. carbonaria* had haplotypes of *C. denticulata*. Haplotype CdH01 was found in two individuals, CdH03 was found in one individual, and the singleton CdH04 was found in one individual. Haplotypes CdH01 and CdH03 were the two most common haplotypes of *C. denticulata*. The presence of these haplotypes within the four *C. carbonaria* individuals could be the result of an ancestral polymorphism maintained in the population during the separation of *C. denticulata* and *C. carbonaria*, or it could indicate a possible hybridization event(s) in the region. However, considering that the maximal within species genetic divergence is approximately 15 times smaller than between species genetic divergence, the coalescent theory suggests it is highly unlikely that the *C. denticulata* haplotypes observed in morphological *C. carbonaria* are due to incomplete lineage sorting (Templeton, 2006). A more plausible explanation is the introgression of *C. denticulata* mtDNA into *C. carbonaria* driven by hybridization between the females of *C. denticulata* and their hybrid offsprings, and males of

C. carbonaria. Occurrence of introgressive hybridization events is higher in areas of sympatry or parapatry than in areas of allopatry (Taylor and McPhail, 2000) and particularly in areas where one species is expanding into the range of another species (see Ballard and Whitlock, 2004 for review), and is often associated with ecologically disturbed areas (Allendorf et al., 2001). Jerozolimski (2005) estimated densities of *C. denticulata* at 25.16-31.44 individuals per km^2, while those of *C. carbonaria* were estimated at 7.87-20.14 individuals per km^2. The hypothesized introgression of mtDNA from the higher-density *C. denticulata* into the expanding lower-density *C. carbonaria* fits the above ecological context of introgression, and is analogous to the well known instance of introgression of coyote mtDNA into wolf populations of Minnesota, an area where low-density wolf populations are expanding into a region currently occupied by coyotes (Lehman et al., 1991).

Unless the *C. carbonaria* genome is dominant over the *C. denticulata* genome, we view introgression as a more likely pattern than bidirectional hybridization since *C. carbonaria* mtDNA haplotypes were not found in morphological *C. denticulata* specimens. Furthermore, none of the putative introgressed individ-

uals exhibited intermediate hybrid morphologies and were unambiguously assignable to *C. carbonaria* (Jerozolimski, pers. obs.). An examination of hybridization phenomena between the two *Chelonoidis* species is outside the scope of this study, since it requires a more intensive sampling of both species at Site number 04 and an analysis of a suite of nuclear markers.

The sympatry of *C. carbonaria* and *C. denticulata* in the Kayapó reserve makes this region of particular interest. The low gene diversity indices found in *C. carbonaria* combined with its high among-population gene flow, bimodal mismatch distribution, as well a significantly negative Tajima and Fu test results suggest that this species may be undergoing a rapid demographic expansion. This expansion could be a response to a likely increase in the availability of suitable habitats for *C. carbonaria* in the local forest due to global climatic changes, large-scale regional deforestation effects, or a combination of both factors. *El Niño* events are know to result in drier, warmer and sunnier climates in the wet tropics (Holmgren et al., 2001) and to cause a reduction of forest soil moisture, and these effects can be more pronounced in ecotonal situations (Sombroek, 2001). Additionally, the large-scale deforestation in the southeastern state of Pará (Fearnside, 2005) might be increasing the strength and duration of the dry season in the study site, an effect already observed in the forests of the Paragominas-Açailândia region (Sombroek, 2001), which could be favoring the populations of *C. carbonaria*.

Furthermore, if *C. carbonaria* is actually expanding its distribution into new areas of drier forest, the sympatry of *C. carbonaria* and *C. denticulata* at the study site might have been intensified due to the rapid expansion of *C. carbonaria* into areas once occupied predominantly by *C. denticulata*. The high displacement capacity of tortoises, highlighted by the radio telemetry data (Jerozolimski, 2005), probably allows them to quickly respond to changes in habitat availability, especially considering that the study area is particularly sensitive to climatic changes.

If the forests of the study area are actually becoming drier, we predict an increase in the abundance of *C. carbonaria* in the next decades. A long term monitoring program of tortoise populations in the study area, associated with a monitoring of climate and forest structure, can potentially increase our knowledge about how wild populations of closely related species with different ecological requirements respond to changes in habitat availability.

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